Mitigation of Methane via Nitrate Feed and Yea-Sacc® Supplementation

Honors Research Thesis

Presented in Partial Fulfillment of the Requirements for Graduation with Honors Research Distinction

Jared Ashworth

Department of Animal Sciences

The Ohio State University

2016

Project Advisor: Dr. Jeffrey Firkins, Department of Animal Sciences

The Ohio State University

Abstract

Dairy cattle are responsible for approximately 128 kg CH_4/head compared with swine and beef (1.5 and 53 kg CH_4/head, respectively). The aim was to feed nitrate to dairy cows to mitigate ruminal CH_4 production by competing with methanogens for H_2 produced during fermentation. *Selenemonas* species of bacteria are stimulated by yeast, so Yea-Sacc® was added to encourage selenomonads to drive DMI and propionate production from lactate. Three squares consisting of 4 lactating Jersey cows each were fed either a diet containing urea or calcium nitrate. Cows were given TMR twice daily with 50 g of a ground corn control or Yea-Sacc® topdress. After a transition period, the cows were on a final diet of 1.5% NO_3 on a DM basis. On the fourth week, rumen, blood, milk, and CH_4 samples were collected. Each period was at least 4 weeks. Rumen samples were collected at 0, 3, 6, and 9 h post feeding, and 6 N HCl was added to stop fermentation for subsequent VFA and ammonia concentrations. CH_4 sampling was taken 8 times over a 3-day period. Blood samples were taken prior to, and 3 h following, feeding for subsequent methemoglobin analysis. Ammonia concentration increased from 10.3 to 12.2 mg/dL of NH_3N ($p=0.11$) when fed NO_3. CH_4 production decreased ($p<0.01$) when cows were fed NO_3. An interaction of NO_3 and Yea-Sacc® ($p=0.01$) was explained by Yea-Sacc® decreasing acetate:propionate without NO_3 but increasing it with NO_3. Total protozoa numbers increased ($p=0.02$), probably explaining the increased butyrate ($p<0.01$). Methemoglobin increased ($p=0.01$) slightly from 0.55 to 1.53%; however, being below 30%, this was not a concern. Future research should focus on increasing DMI. Lower dosages or other NO_3 forms might improve palatability. Determination of bacterial DNA results would help explain if *Selenomonas* species increased when NO_3 and Yea-Sacc® were fed.

Introduction

In 2010, the livestock sector contributed to approximately 7.3% of the global greenhouse gas emissions, whether it was by generating carbon dioxide (CO_2), methane (CH_4), and/or nitrous oxide; this statistic was either directly resulting from enteric fermentation and manure management or indirectly from feed-production activities and conversion of forest into pasture (Hristov *et al.* 2013). Within this sector, recent research has focused mainly on dairy cattle, for they produce approximately 128 kg CH_4/head per year, contributing to 17 and 3.3% of CH_4 and greenhouse gases, respectively, from enteric fermentation (Knapp *et al.* 2014). Thus, a decrease in the amount of enteric CH_4 could substantially decrease the amount of greenhouse gases associated with milk production. The most practical and widely used techniques for measuring enteric CH_4 production are the SF_6 tracer gas method and, more recently, an Automated Head-Chamber System (GreenFeed) that monitors CH_4 and CO_2 mass fluxes from the breath and eructation gas of ruminants (Hristov *et al.* 2015).

When it comes to mitigating enteric CH_4 production, any dietary strategy must show a persistent decrease; this is an absolute requirement at being successful in lessening greenhouse gas emissions in dairy cattle (van Zijderveld *et al.* 2011). Methane gas is a natural by-product of microbial fermentation of carbohydrates in the rumen. Due to the dairy cow's anaerobic fermentation, glucose produced from microbial polysaccharide decomposition is quickly converted to CO_2, which in turn is reduced to CH_4 with aqueous H_2 via methanogenic prokaryotes present in the rumen (Hristov *et al.* 2013). The high affinity by methanogens for H_2 keeps concentration of aqueous H_2 very low and therefore pulls H_2 production in a process that would be otherwise thermodynamically unfavorable. This process of H_2 production removes

excess reducing equivalents from NADH and regenerates NAD^+, which is essential for the continuation of anaerobic rumen fermentation and microbial growth (van Zijderveld *et al.* 2011).

The addition of nitrate (NO_3) in the diet has shown promise in decreasing enteric CH_4 production. Anaerobic NO_3 reduction is thermodynamically more favorable (ΔG^{0t} = -600 kJ/mol) than CO_2 reduction (ΔG^{0t} = -136 kJ/mol) and the reduction of other electron acceptors available in the rumen's environment (Latham *et al.* 2016). This is because NO_3 is reduced in a dissimilatory fashion, consuming more electrons, at the expense of methanogenesis, when being reduced to nitrite (NO_2) and furthermore to ammonia (NH_3) (Latham *et al.* 2016). With the presence of NO_3 in the rumen, aqueous H_2 is redirected from methanogenic archaea, thereby reducing the production of CH_4 gas. Methemoglobinemia, a condition caused by the oxidation of the ferric iron in hemoglobin—rendering the molecule incapable of oxygen transport—may occur due to the introduction of NO_3 in the diet; thus, a gradual transition period is necessary to allow the rumen microbes to adapt and increase their capability to reduce NO_3 (van Zijderveld *et al.* 2011).

Nitrate is also beneficial to bacteria present in the rumen. Those of the *Selenomonas* species are the best characterized NO_3-reducing bacteria; by reducing NO_3 to NH_3, these microorganisms gain an energetic benefit via a more effective disposal of electrons and are therefore considered to be more efficient at recycling NAD^+ via oxidation of NADH (Latham *et al.* 2016). Yea-Sacc®, a live-strain yeast culture of *Saccharomyces cerevisiae*, is known to stimulate *Selenomonas* species and improve production efficiency, and digestibility of dry matter, crude protein, and hemicellulose when added to the diet (Callaway *et al.* 1997). Also, ruminal bacterial numbers have shown to increase while lactate concentrations have decreased when Yea-Sacc® is introduced.

The present study aimed to feed NO_3 to dairy cows to mitigate ruminal CH_4 production by allowing NO_3 reducers to outcompete methanogens for aqueous H_2 produced during enteric fermentation. The reduction of either CO_2 or NO_3 should help maintain acetate production because it is coupled with H_2 production by those microbes that link NADH oxidation to electron disposal as H_2 (typically using ferredoxin as a cofactor). *S. ruminantium* activity and H_2 uptake has increased with live-yeast supplementation, so Yea-Sacc® was added to encourage *S. ruminantium* to assist in reducing NO_3 fully to NH_3 (Miao, 2013). The study's hypotheses were:

- Feeding NO_3 would increase NH_3 production and decrease CH_4 production but might be unpalatable.
- Because Yea-Sacc® often enhances DMI and stimulates NO_3 reducers, the combination of NO_3 and Yea-Sacc® would further decrease CH_4 while maintaining milk production.

Materials and Methods

The experimental design was that of a replicated 4x4 Latin square with 12 lactating Jersey cows (8 multiparous and non-cannulated; 4 primiparous and ruminally cannulated). In a 2x2 factorial arrangement, treatments were assessed with diets containing urea or calcium nitrate, both being isonitrogenous. Before the treatments were provided to the cows, a 3-week transition period was allotted in order for microbial groups to adapt to the new diets. Afterwards, the cows were on a final diet of 1.5% NO_3 on a DM basis. Each period was at least 4 weeks. Cows were given a total mixed ration (TMR) twice daily with 50 g of a ground corn control or Yea-Sacc® topdress. Fresh water was provided ad libitum. On the fourth week of each period, rumen, blood, milk, and CH_4 measurements were taken.

Rumen Sampling

Rumen contents were subsampled from various locations in the rumen of each of the 4 cannulated cows at 0, 3, 6, and 9 h post-feeding. Approximately 1 L of rumen fluid was obtained. Approximately 250 mL of this was subsampled and mixed in a blender for future DNA analysis. The remaining sample was filtered through cheesecloth into a 250-mL bottle and its pH was recorded immediately. In a whirlpack bag, 50 mL of rumen fluid was mixed with 3 mL of 6 N HCl in order to stop the fermentation process. The bag was closed and inverted to mix the sample thoroughly and placed in -80°C for later determination of VFA and NH_3 concentrations. A total of 30 mL of rumen fluid was poured into 2 Falcon tubes, 15 mL of sample in each, and put on ice in order to later determine NO_3 and NO_2 concentrations. The tubes were spun in a centrifuge at 15,000 x *g* for 15 minutes at 4°C. The supernatant was removed and then stored in 3 microtubes in -80°C. In a Falcon tube containing 6 mL of formalin, 6 mL of rumen fluid was added and stored on a shelf for protozoal counts.

Methane Emission Collection

An automated system (GreenFeed) was used to collect CH_4 and CO_2 measurements. Methods of using the GreenFeed were similarly followed as those described by Hristov *et al.* (2015). Gas samples were collected 8 times over a 3-day period in order to have a representative collection throughout the day without disturbing the cows' laying and feeding times. These samples were collected from the 8 intact cows to minimize CH_4 and CO_2 losses associated with cannulated cows.

Prior to sampling for each period, the GreenFeed was calibrated using a span gas, 0.15% CH_4 and 0.98% CO_2, and a zero gas, 100% N_2. Recovery of CO_2 was also conducted 3 times prior to each sample period. The GreenFeed was allowed to warm up for at least 30 minutes.

The cows were then baited into the GreenFeed using a portion of their grain mix for that day. Grain was continuously being dropped into the GreenFeed feed pan for 5 minutes in order to keep their muzzle within range to measure CH_4 and CO_2 expired. After 5 minutes, the GreenFeed was moved away from the cows and flushed with air from a box fan for 2 minutes before being placed in front of another cow. The whole process took approximately 1.2 h to sample all 8 cows. CH_4 and CO_2 measurements were uploaded from GreenFeed online to be analyzed and adjusted by C-Lock Inc.

Blood Sampling

Blood was collected from all 12 cows, via either tail or jugular veins. For each cow, 2 heparinized vacutainer vials were filled with blood, placed on ice, and transported to The Ohio State University's College of Veterinary Medicine for methemoglobin analysis. Only a small aliquot was needed for this analysis, so the remaining blood was processed at the Animal Sciences Building. The blood is being stored for later analysis for percent NO_3 and percent NO_2.

Data Analysis

For p-values less than or equal to 0.05, the results are considered to be statistically significant. P-values between 0.05 and 0.10 signify a trend in the data; however, if the SEM is relatively high, p-values ranging from 0.10 to 0.15 are to be considered in this experiment as acceptable and therefore signify a trend.

Results

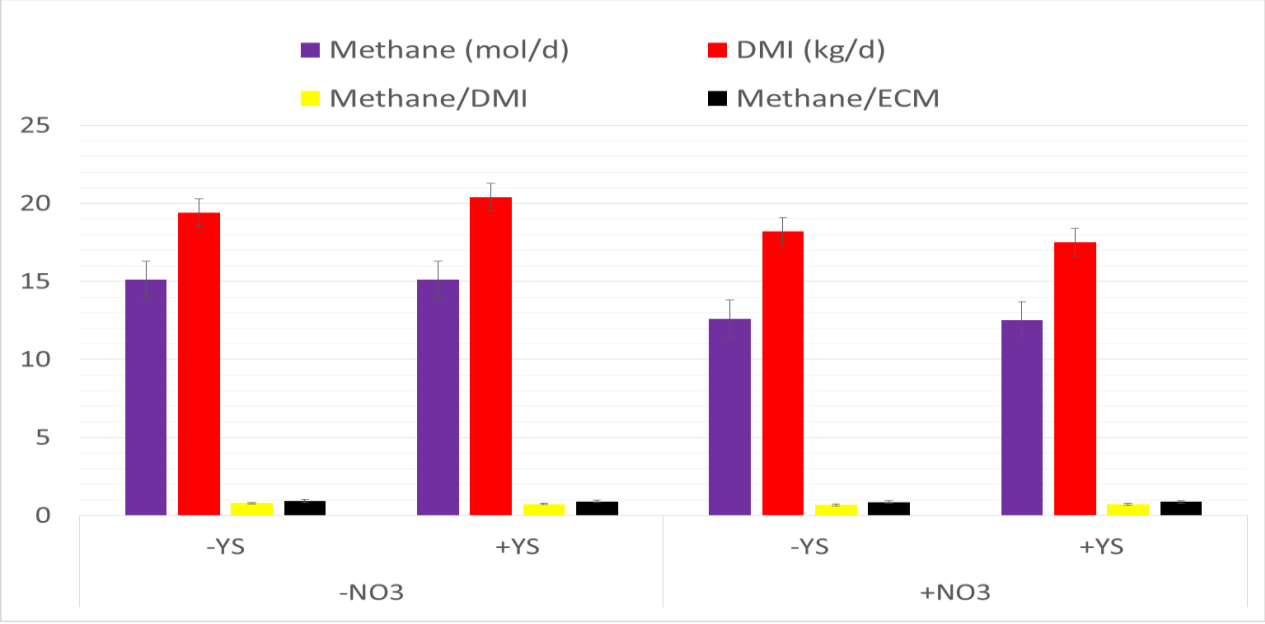
Table 1: Volatile Fatty Acid Concentrations

	-NO_3		+NO_3			P values for contrasts		
	-YS	+YS	-YS	+YS	SEM	NO_3	YS	NO_3*YS
Total VFA, mM	98.3	92.1	94.8	90.8	4.8	NS	0.11	NS
VFA, mol/100 mol								
Acetate	63.6	61.5	61.9	63.7	1.3	NS	NS	< 0.01
Propionate	20.1	22.4	20.1	18.5	1.3	< 0.01	NS	< 0.01
Butyrate	11.6	11.7	14.2	13.9	0.5	< 0.01	NS	NS
Isobutyrate	0.98	1.07	0.84	0.98	0.07	0.11	0.14	NS
Isovalerate	1.44	1.68	1.43	1.58	0.20	NS	0.01	NS
Valerate	1.40	1.53	1.46	1.37	0.13	NS	NS	0.13
BCVFA	3.82	4.28	3.73	3.93	0.25	0.10	0.02	NS
Acetate:propionate	3.11	2.88	3.13	3.47	0.23	<0.01	NS	0.01

YS=Yea-Sacc®, VFA=Volatile Fatty Acids, NO3=Nitrate, BCVFA=Branched-Chain Volatile Fatty Acids

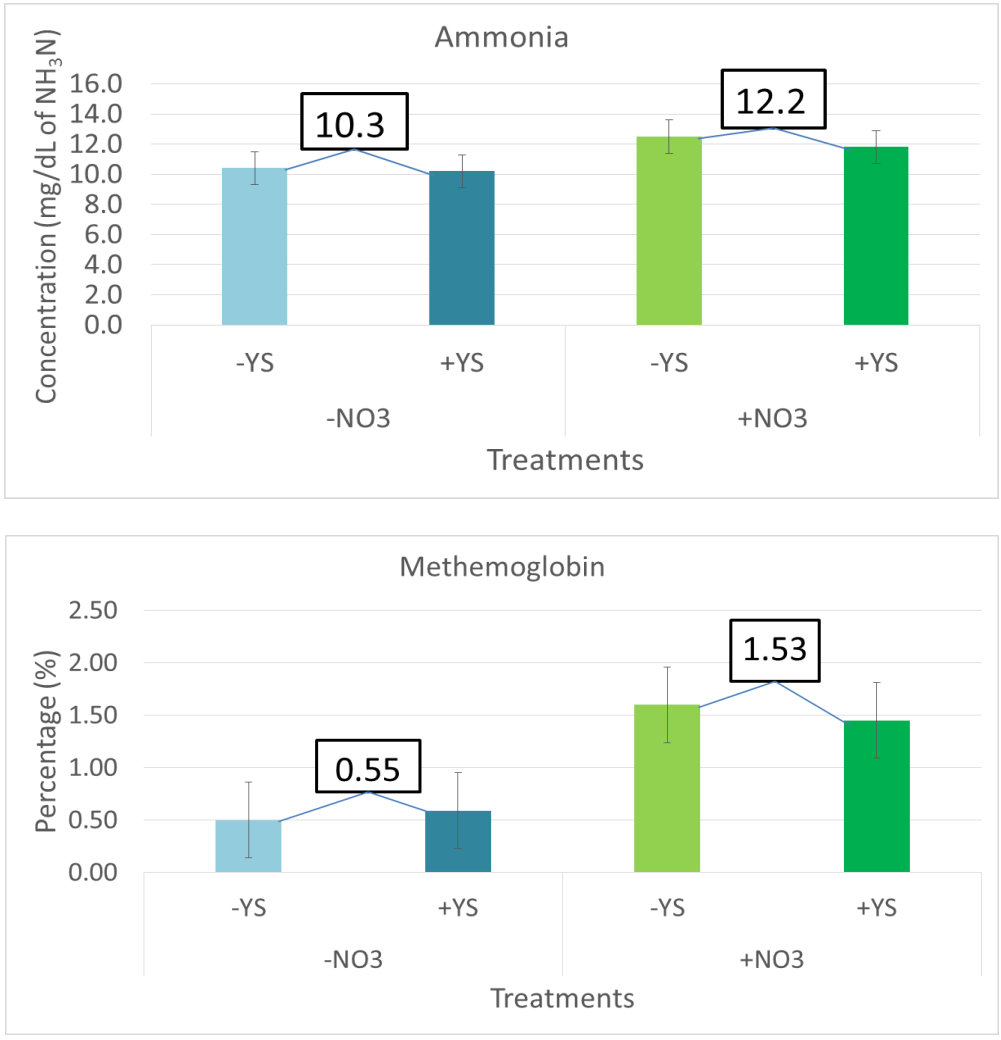
No effect of time was observed across treatments; data are expressed as treatment means. Total VFA concentration decreased with Yea-Sacc® (main effect). The combination of NO_3 and Yea-Sacc® had the highest acetate and the lowest propionate concentrations, resulting in the highest acetate:propionate ratio (interaction; p=0.01; Table 1).

Figure 1: Methane Production and Dry Matter Intake



Data expressed as treatment means ± SE. Methane production (15.1 vs. 12.6 mol/d) and DMI (19.9 vs. 17.8 kg/d) decreased when fed NO_3 (main effect; $p<0.01$). Even though not significant, nitrate tended to decrease methane/DMI (main effect; $p=0.14$). Yea-Sacc® treatment did not affect CH_4 production or DMI.

Figure 2: Ammonia Concentrations and Methemoglobin Percentages



No interaction was found with time for NH_3 concentrations; Figure 2 means are expressed over time. There was a numerical difference that might be related to the decreased DMI. Methemoglobin increased slightly (0.55 vs. 1.53%) when fed NO_3 (main effect; $p=0.01$); however, being below 30%, this was not a concern.

Figure 3: Protozoa Concentration

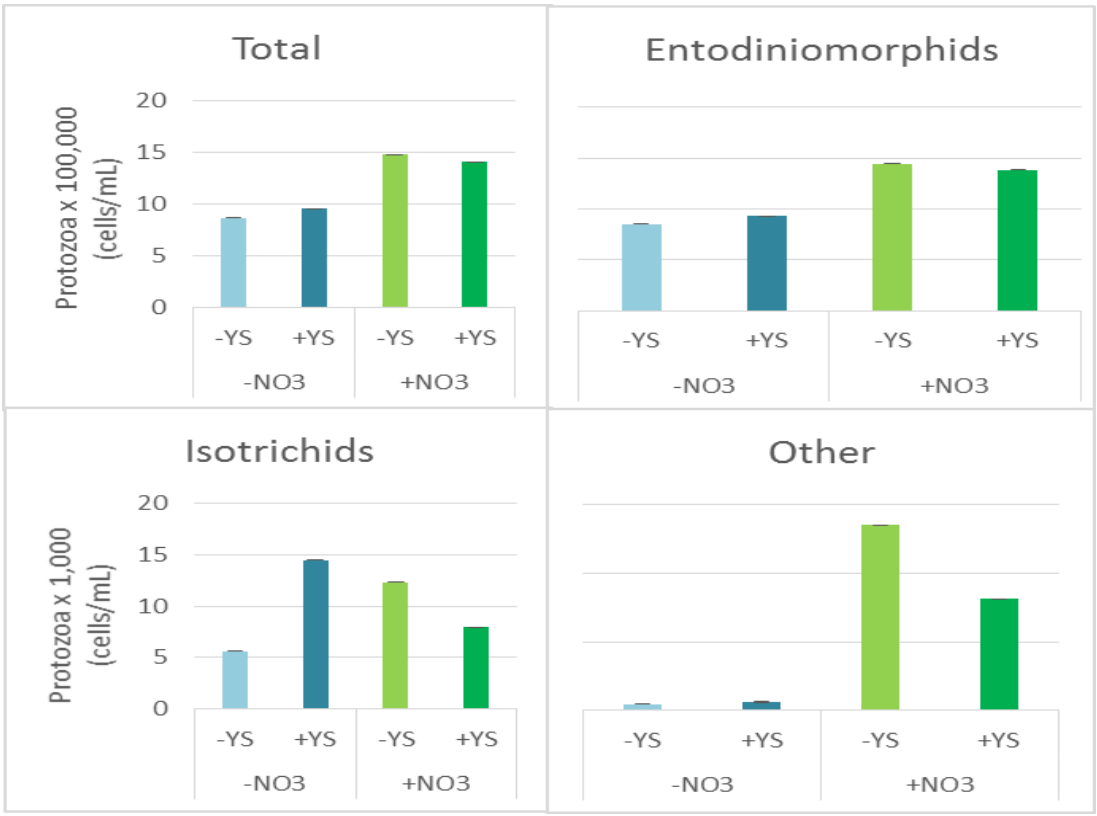


Figure 3 data had no effect over time. Total protozoa numbers increased when fed NO_3 (main effect; $p=0.02$), probably explaining the increased butyrate (main effect; $p<0.01$; Table 1). Nitrate increased the total, entodiniomorphid, and other protozoa numbers ($p<0.03$). There was a treatment interaction for isotrichid concentration ($p=0.11$).

Discussion and Conclusions

Dietary NO_3 has been of interest to ruminant nutritionists because of its effect on mitigating enteric CH_4 emissions in dairy cows. Nitrate can be fully reduced to NH_3, which can then be directly used for rumen microbial protein synthesis (Yang *et al.* 2016). The reduction is thermodynamically more favorable than reducing CO_2 to CH_4 and can be linked to ATP synthesis by electron transport-linked phosphorylation in some microbial species, ultimately increasing the growth yield of NO_3-reducing organisms, such as *S. ruminantium* (Miao, 2013).

However, NO_3 tastes bitter, which lowers palatability of NO_3-based diets and may cause lower feed intake (Yang *et al.* 2016). In our study, there was a decrease in DMI (19.8 vs. 17.8 kg/d) when cows were fed NO_3 (main effect; $p<0.01$). Consequently, total VFA concentrations were reduced. There was a trend of Yea-Sacc® reducing total VFA concentrations even further, from 96.6 to 91.5 mM (main effect; $p=0.11$), which differed from expectation. The combination of nitrate and Yea-Sacc® had the highest acetate and the lowest propionate concentrations, resulting in the highest acetate:propionate ratio ($p=0.01$).

Enteric CH_4 production decreased (15.1 vs. 12.6 mol/d) when cows were fed NO_3, which supported proof of concept (main effect; $p<0.01$). Nitrate tended to reduce methane/DMI (0.77 vs. 0.71 mol/kg; main effect; $p=0.14$ and SEM was 0.056), but the Yea-Sacc® treatment did not affect CH_4 production or DMI, which differed from expectation. We accepted a trend with a p-value from 0.10 to 0.15 because of the larger SEM value. As CH_4 production decreased, NH_3 concentrations increased (10.3 vs. 12.2 mg/dL of NH_3-N) when fed NO_3 (main effect; $p=0.11$).

When NO_3 is consumed in relatively large quantities, its reduction pathway can cause methemoglobinemia as NO_2 accumulates in the rumen and is absorbed into the blood (Lee and Beauchemin, 2014). Nitrite in the blood binds to red blood cells and changes the ferrous form of hemoglobin to the ferric form, making it incapable of carrying oxygen. Depending on the degree of methemoglobinemia, cows can manifest symptoms such as depressed feed intake and production, no weight gain, susceptibility to infection, reproductive failure, respiratory distress, and even death (Lee and Beauchemin, 2014). In this study, methemoglobin percentages increased slightly (0.55 vs. 1.53%) when fed NO_3, which signified that the NO_2 from that treatment was being absorbed (main effect; $p=0.01$); however, being below 30%, this was not a concern.

The rumen contains a vast array of protozoa, anaerobic fungi, anaerobic bacteria, and archaea. This study focused on protozoa because they have been known to play a crucial role in NO_3 and NO_2 reduction; these microorganisms have the ability to use NO_3 as an electron acceptor and thus decrease enteric CH_4 emissions (Lin *et al.* 2011). However, in some studies, the feeding of NO_3 or NO_2 was shown to be toxic to protozoa (Lee and Beauchemin, 2014). With this being said, more research is needed in order to determine the effect of NO_3 on rumen protozoa on mitigating enteric CH_4 emissions. In this study, total protozoa numbers increased when fed NO_3 (main effect; $p=0.02$), probably explaining the increased butyrate concentration (main effect; $p<0.01$; Table 1). Nitrate also increased the entodiniomorphid and other protozoa numbers ($p<0.03$). There was a treatment interaction for isotrichid concentration ($p=0.11$).

Future research should look into lower dosages of NO_3 or feeding a different source of NO_3 to improve palatability in order to increase DMI and reduce the acetate:propionate ratio. Nitrate in feed successfully outcompeted methanogens and, in turn, was reduced to NH_3. Nitrate competed with CO_2 for electrons from hydrogen. Methemoglobin increased but to a percentage that was not a concern. Determination of archaeal and bacterial DNA results would help explain if *Selenomonas* and other species increased when NO_3 and Yea-Sacc® were fed. Based on few interactions detected, Yea-Sacc® had a minimal role in attenuating ruminal methanogenesis. This type of in vivo research has helped explain how the microbiome structure is changed after a major disruption of inter-species hydrogen transfer.

References

Callaway, E. S., & Martin, S. A. (September 01, 1997). Effects of a Saccharomyces cerevisiae Culture on Ruminal Bacteria that Utilize Lactate and Digest Cellulose. *Journal of Dairy Science, 80,* 9, 2035-2044.

Hristov, A. N., Oh, J., Giallongo, F., Frederick, T., Weeks, H., Zimmerman, P. R.,…Branco, A. F. (2015). The Use of an Automated System (GreenFeed) to Monitor Enteric Methane and Carbon Dioxide Emissions from Ruminant Animals. *Journal of Visualized Experiments : JoVE,* (103), 52904.

Hristov, A. N., J. Oh, J. L. Firkins, J. Dijkstra, E. Kebreab, G. Waghorn, H. P. Makkar, A. T. Adesogan, W. Yang, C. Lee, P. J. Gerber, B. Henderson, and J. M. Tricarico. 2013. Special topics--Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. J. Anim. Sci. 91:5045-5069.

Knapp, J.R., Laur, G.L., Vadas, P.A., Weiss, W.P., Tricarico, J.M. (2014) Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions, Journal of Dairy Science, Volume 97, Issue 6, 3231-3261.

Latham E.A., Anderson R.C., Pinchak W.E. and Nisbet D.J. (2016) Insights on Alterations to the Rumen Ecosystem by Nitrate and Nitrocompounds. *Front. Microbiol*. 7:228.

Lee, C., & Beauchemin, K. A. (January 01, 2014). A review of feeding supplementary nitrate to ruminant animals: nitrate toxicity, methane emissions, and production performance. *Canadian Journal of Animal Science, 94,* 4, 557-570.

Lin M., Schaefer D. M., Guo W. S., Ren L. P., Meng Q. X. (2011). Comparisons of in vitro nitrate reduction, methanogenesis, and fermentation acid profile among rumen bacterial, protozoal and fungal fractions. *Asian Australas. J. Anim.* 24 471–478.

Miao, L., Jing, L., Xia, Q., Peng, L., Wen-Kui, Z., Guo-Wen, L., & Xiaobing, L. (April 01, 2013). Effect of Different Yeasts on Selenomonas ruminantium Utilizing Lactate in vitro. *Indian Journal of Animal Research, 47*, 2.

van Zijderveld, S. M., Gerrits, W. J. J., Dijkstra, J., Newbold, J. R., Hulshof, R. B. A., & Perdok, H. B. (August 01, 2011). Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. *Journal of Dairy Science, 94*, 8, 4028-4038.

Yang, C., Rooke, J. A., Cabeza, I., & Wallace, R. J. (2016). Nitrate and Inhibition of Ruminal Methanogenesis: Microbial Ecology, Obstacles, and Opportunities for Lowering Methane Emissions from Ruminant Livestock. *Frontiers in Microbiology*, *7*, 132.